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Cytogenetic evidence for a complex of species within the taxon *Anopheles maculatus* (Diptera: Culicidae)

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Two largely independent studies of chromosomes from natural populations of *Anopheles maculatus* provide evidence for several genetic species within the taxon. (1) Polytene chromosome variation shows four different rearrangements of arm 2 and three rearrangements of the X chromosome. There is strong evidence for three species. Two allopatric populations represent either dramatic geographic variation for two independent inversion systems within one of the genetic species, or represent two additional species. Their species status remains unresolved by this work. (2) Heterochromatic variation occurs in both X and Y chromosomes as revealed by Giemsa-banding of mitotic chromosomes from larval brains. The distribution and association of these various sex chromosomes give further evidence of a species complex. A preliminary correlation of these two kinds of chromosomal variation is given.

KEY WORDS—Polytene chromosomes – Giemsa-banded mitotic chromosomes – *Anopheles maculatus* species complex.

CONTENTS

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INTRODUCTION

Anopheles (Cellia) maculatus Theobald occurs throughout the Oriental zoogeographic region. It is highly variable in both its morphology and ability to transmit malaria (Reid, 1968). Studies by Christophers (1931), Reid *et al.* (1966) and Reid (1968) attempted to unravel the status of the seven synonyms, morphological forms or varieties of *maculatus*, but to date, the morphological, ecological and vectorial variability observed in this taxon remains unresolved. Reid (1970) suggested that *maculatus* may represent another complex of species having little or no morphological differentiation.

Anopheles maculatus has excellent polytene chromosomes in the ovarian nurse cells of half-gravid females. Interspecific relationships of *maculatus* and its close relatives within the series Neocellia are known (Green, 1982a) from the rearrangements found in the polytene chromosomes. Green (1982b) presented data (included in this report) from polytene chromosomes which showed that there are two, possibly three species, within *maculatus* in Thailand. Nothing is known about heterochromatin variation in the mitotic chromosomes of these species. In two largely independent studies we used these sources of chromosomal variation to determine if they might provide evidence of genetic species within the taxon. Our results are derived from data obtained from natural populations in Thailand, one from Malaysia and a very small sample from the Philippines, and three laboratory colonies (two from Thailand and one from peninsular Malaysia).

MATERIALS AND METHODS

The locality and size of samples are documented in Table 1. Material for analysis of polytene chromosomes was collected direct from nature. Mitotic chromosomes were prepared from late third- to early fourth-instar larvae of isolated clutches of eggs from wild-caught females. Cytological techniques used have been documented for polytene (Green & Hunt, 1980) and G-banded mitotic chromosomes (Baimai, 1975). The various arrangements of the polytene chromosomes are presented in Fig. 1, and those for mitotics in Fig. 2. The presentation of the rearrangements shown in Fig. 1 follows the method of Green (1982c). Briefly, the *maculatus* data have been incorporated into a single scheme of relationship with near relatives (Coluzzi *et al.*, 1970, 1973; Green 1982a) based on their polytene-chromosome rearrangements. *Anopheles stephensi* is close to the arbitrary standard arrangement (*superpictus*, suggested by Coluzzi *et al.*, 1970). Arm $2a+^{b}+^{c}+^{d}$ in Fig. 1 is from *stephensi* to which the homologies of the *maculatus* arrangements are referred. Inversions 2abcd on the *stephensi* arm 2 (Fig. 1) are documented by Coluzzi *et al.* (1970, 1973).

Certain crosses were made in the laboratory between the F_1 progeny of wild-caught females.

RESULTS AND DISCUSSION

Polytene chromosome arrangements were distributed into six distinct forms (A to F) summarized in Fig. 3 where black squares indicate the occurrence of the putative derived alternative for an inversion (e.g. 2p) and white squares

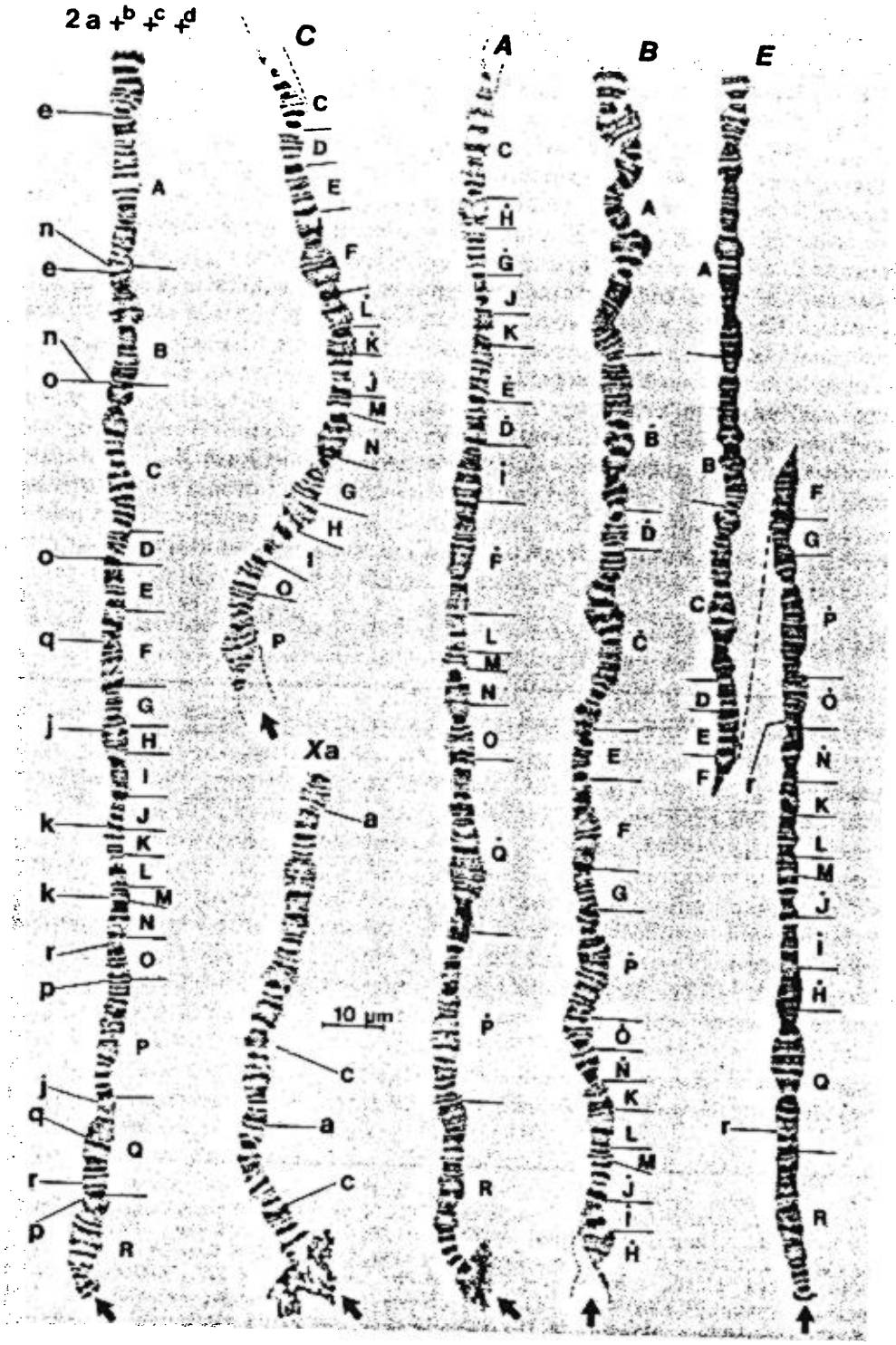
show the occurrence of the probable ancestral alternative (e.g. $2+^{p}$). However, this is not true for the X chromosome since interspecific homologies in terms of inversions are not known in Neocellia (Green, 1982a), i.e. which alternative is probably ancestral and which derived for Xa and Xc is unknown. Form D has the following formula 2aq (i.e. homosequential with *stephensi* except for the fixation of 2q), Xa. The complexes '2C' and '2A' refer to the unique rearrangements in these forms, i.e. in Fig. 1 *C* blocks L through O in form C and Fig. 1 *A* blocks H through F in form A. There are several possible inversion sequences that may be postulated for these two complexes, any of which might have occurred in actual fact. This is because there are evidently several common breakpoints to two or more inversions. There is no useful information contained in presenting one of these several and equally likely sequences of hypothetical inversions. The block relationships shown in Fig. 1 *C* and *A* are the primary data. The Wat Pratart sample shows that forms A and B are distinct genetic species because there were no heterozygotes for complex '2A', 2p and 2j. Furthermore, there was only one heterozygote for $2no/+^{no}$ which occurred together with $2jk+^{p}$ and in the absence of complex '2A'. Similarly, there is a massive relative deficiency of heterozygotes for 2k; two occurred within form B. The status of form C is reasonably certain despite the small samples since it is homozygous for complex '2C', $2+^{p}$, and $X+^{a}$, but fixed for those inversion alternatives, $2+^{j}+^{k}+^{no}$, that do not occur, or are rare, in species B. Should form D prove to

Table 1. Localities, sample sizes and distribution of chromosomal variants in natural populations of *Anopheles maculatus*

	Mitotic chromosomal forms				Polytene chromosomal forms					
Location*	I X_1Y_2	II $X_2X_3Y_3$	III X_1Y_1	IV $X_2X_3Y_4$	A	B	C	D	E	F
1								4		
2	10	5	1		113	38	2			
3					7	1				
4					7	1				
5	3	17		1	11					25
6		13		8					32	
7		2		6					8	
8	1	14				2				
9			2		12	2	6			
10					10	8	1			
11		5		3					27	
Colonies										
12				†					†	
13				†					†	
14		†				†				

*Key to localities: 1, Montalban, near Manila, the Philippines; 2, Wat Pratart, Kanchanaburi Province, 14°25'N, 99°07'E; 3, near Mae Hong Son, 19°12'N, 97°54'E; 4, near Rong Kwang, Phrae Province, 18°26'N, 100°24'E; 5, near Nakhon Nayok, 14°19'N, 101°18'E; 6, near Phato, Ranong, 9°46'N, 98°41'E; 7, near Phangnga, 8°31'N, 98°33'E; 8, near Chantaburi, 12°45'N, 102°11'E; 9, Sam Larng, Kanchanaburi Province, 14°40'N, 99°21'E; 10, Ban Chaang, Chiengmai Province, 19°11'N, 98°52'E; 11, Genting Highlands, near Kuala Lumpur, 3°15'N, 101°44'E; 12, Nakhon Nayok; 13, Kuala Lumpur (ex. Institute of Medical Research, Kuala Lumpur, Malaysia); 14, Hoi Kuum, Cholburi Province. Localities 2–10, 12 and 14 are in Thailand.

† Indicates the chromosome types found in the colonies.


2a +b +c +d
C
A
B
E
Xa
10 µm

be the only *maculatus* in the Philippines, and so allopatric to all other forms or species than its status will have to be determined from the indirect evidence provided by laboratory hybridization studies. A decision about its specific status requires further study.

The status of forms E and F remain unresolved by this work. They might represent geographic variation within species B or represent distinct species homosequential with species B. What distinguishes them are dramatic differences in inversion frequencies between each other and each with species B. These are:

	N	$X+^a$	Xc	$2+^n$	$2+^o$	$2+^k$	$2+^r$
B	38	0.04	0.00	0.01	0.01	0.04	0.72
E	32	0.81	0.00	0.98	0.98	0.17	0.95
F	25	0.16	0.84	0.90	0.84	0.06	0.92

Arm 2no occurred in a single heterozygous individual in form E; $2+^n+^o$ in a single specimen of species B. In form F, two individuals were heterozygous for 2n and homozygous for $2+^o$; one heterozygous for 2o and homozygous for 2n, and three were double heterozygotes. It seems likely that these two inversions are linked; not surprising, since they are very close together on the chromosome, if not immediately contiguous with each other as Fig. 1 suggests. If the forms E and F are different species from species B, then their sympatric occurrence with B would show massive relative deficiencies of heterozygotes for those inversions with frequency differences seen above. On the other hand should these frequency differences represent geographic variation within species B, then further sampling should show a dramatic cline between Ranong and Wat Pratart, 525 km apart, in the case of form E. The geographic/ecological situation between B/E and form F is more complex since they are separated by the Chao Phraya River basin from which *maculatus* is absent. Detailed geographic sampling should resolve the status of forms E and F.

Heterochromatin variation in the mitotic chromosomes occurred as three different X chromosomes (X_1–X_3) and four different Y chromosomes (Y_1–Y_4), which are shown in Fig. 2. These chromosomes occur in four different combinations within individual families, shown in Table 1 as forms I, II, III, and IV. Since the progeny broods of wild-caught females are scored for variation, it is possible to determine both parental genomes. The various X chromosomes assorted into two groups, forms I+III and II+IV, Table 1. The data from Wat Pratart, Nakhon Nayok, and Chantaburi (Table 1) reveal two separate species since in no case were heterozygous females seen for X_1, though they were for the other two X chromosomes. Notice that this evidence is independent of the polytene chromosome data. A small sample of families

Figure 1. Rearrangements of ovarian polytene chromosomes found in natural populations of *Anopheles maculatus*. Arm $2a+^b+^c+^d$ at the extreme left comes from *stephensi*. *C* is the complex rearrangement, arm 2, in species C; the rest of the arm (not shown here) is homosequential with *stephensi*. *A* is the arm 2 arrangement in species A, the missing distal part is homosequential with *stephensi*. *B* is the common arrangement of arm 2 in species B, the missing centromeric end is homosequential with form E. *E* is the arm 2 arrangement commonly found in form E and F, it also occurs rarely in species B. In all cases the arrows indicate the centromeres of the arms. Block designations indicate homologies between all arm 2 arrangements and a dot over block designations indicates them to be mirror images of normally designated blocks. Note that inversion Xa must be made on the figured arm before Xc may be derived.

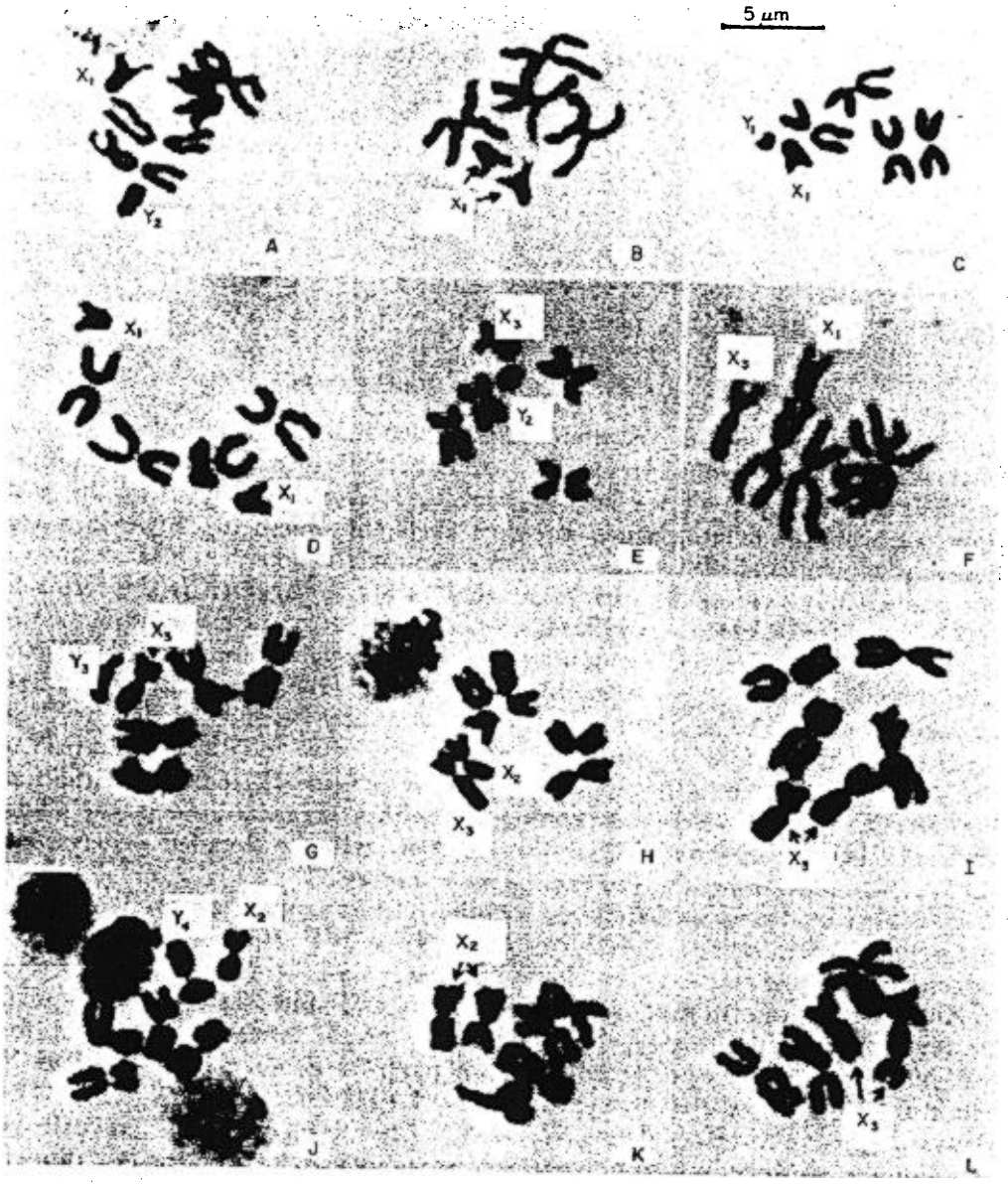


Figure 2. Heterochromatic variation in sex chromosomes from natural populations of *Anopheles maculatus* as seen in mitotic metaphase sets from larval neuroblasts. A, Form I male; B, Form I female; C, Form III male; D, Form III female; E, male and F, female F_1 hybrids from the cross Form I male X Form II female; G, Form II male; H, heterozygous Form II female; I, homozygous Form II female; J, Form IV male; K and L, the two homozygotes of Form IV females.

suggests that mitotic form I (see Table 1) is associated with species A (12 families), and that mitotic forms II and IV are associated with species B and forms E and F (4, 16 and 8 families respectively) and form III with species C (two families). That associated data from Wat Pratart (Table 1) shows similar frequencies for polytene and mitotic variation further supports the correlation of the two in the case of species A and B. The Y chromosome variation is

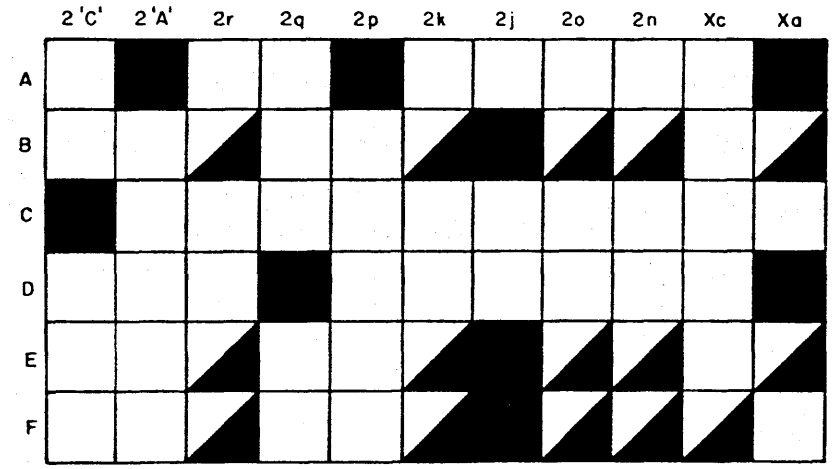


Figure 3. Summary of the distribution of variation for polytene chromosomes in the forms and species of the *Anopheles maculatus* complex. Black squares indicate the presence of the inversion alternatives marked on the figure whilst white squares indicate possession of the standard alternatives, X+ᵃ etc. Half squares show that both alternatives of a particular inversion occurs in a form or species but do not indicate relative frequencies. Note that 2'A' and 2'C' refer to the two complexes of fixed inversions unique to species A and C respectively (see text and Fig. 1 *C* and 1 *A* for explanation).

troublesome in the diagnosis of genetic species since direct frequencies of their occurrence in individuals gives no indication as to whether they are polymorphic within a single species or if they reflect two or more species. This is because they are always in the hemizygous condition. Both Y_3 and Y_4 are associated with species B and forms E and F; however, they show geographic variation. Chromosome Y_4 does not occur (or is rare) at Wat Pratart and Chantaburi, is rare at Nakhon Nayhok (relative frequency 0.06, 18 families), and is more common at Ranong (0.38, 21 families) and Genting Highlands, Malaysia (three of eight families). In *Drosphila* comparable variants in Y chromosomes very rarely, if ever, occur together in the same populations of the same species (Dobzhansky & Epling, 1944; Miller & Roy, 1964; Baimai, 1969; Baimai *et al.*, 1983). Therefore we wonder if their occurrence together in nature within *maculatus* might indicate different species. One could test the association of the different Y chromosomes with other genetic variation where 'linkage disequilibria' between the Y's and the other variation would indicate different species. It is unfortunate that we did not score the frequencies of the different X chromosomes in sibling females of males having different Y chromosomes, but simply noted their presence or absence in families.

A second and more direct test involves the use of asynapsis/synapsis of the polytene chromosomes. In no case of any wild-caught females have we seen asynapsis of the polytene chromosomes, so we could use the presence of asynapsis in laboratory-produced offspring as indicating interspecific hybrids where wild-caught females had been collected together. Indeed such data provided some of the first direct evidence for the specific status of the freshwater members of the *Anopheles gambiae* group of species (Paterson, 1965). Therefore we crossed the F_1 of cytologically-typed familes from females caught together at Phato and which showed Y_3, and Y_4 (three families of each Y chromosome type crossed in three pair-wise combinations). The F_2 females showed total synapsis

of their ovarian polytene chromosomes. In crosses between B (Wat Pratart) × F (Nakhon Nayok), polytenes showed complete synapsis even in the case of the complex mechanical configuration taken by heterozygotes of Xa/Xc (a single family of each was used). Thus, available evidence suggests that these Y chromosomes are polymorphic within a species, at least at Phato, however, we stress the adjective 'available' since synapsis does not necessarily prove conspecificity of two forms.

Since submission of this report a fourth species, G, has been found in Thailand. Details are given in:
Green, C. A., & Baimai, V., 1984. Polytene chromosomes and their use in species studies of malaria vectors as exemplified by the *Anopheles maculatus* complex. In V. L. Chopra, B. C. Joshi, R. P. Sharma & H. C. Bansal (Eds), *Genetics: New Frontiers. Proc. XV International Congress of Genetics. 3:* 89–97. New Delhi, Bombay, Calcutta: Oxford and IBH Publishing Company.

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